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Dynamic expression of DNMT3a and DNMT3b isoforms during male germ cell development in the mouse

Sophie La Salle [a], Jacquetta M. Trasler [a,b,*]

[a] *Department of Pharmacology and Therapeutics and the Montreal Children's Hospital Research Institute, McGill University, Montreal, QC Canada H3H 1P3*
[b] *Departments of Pediatrics and Human Genetics, McGill University, Montreal, QC Canada H3H 1P3*

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Abstract

In the male germ line, sequence-specific methylation patterns are initially acquired prenatally in diploid gonocytes and are further consolidated after birth during spermatogenesis. It is still unclear how DNA methyltransferases are involved in establishing and/or maintaining these patterns in germ cells, or how their activity is regulated. We compared the temporal expression patterns of the postulated de novo DNA methyltransferases DNMT3a and DNMT3b in murine male germ cells. Mitotic, meiotic and post-meiotic male germ cells were isolated, and expression of various transcript variants and isoforms of *Dnmt3a* and *Dnmt3b* was examined using Quantitative RT-PCR and Western blotting. We found that proliferating and differentiating male germ cells were marked by distinctive expression profiles. Dnmt3a2 and Dnmt3b transcripts were at their highest levels in type A spermatogonia, decreased dramatically in type B spermatogonia and preleptotene spermatocytes and rose again in leptotene/zygotene spermatocytes, while Dnmt3a expression was mostly constant, except in type B spermatogonia where it increased. In all cases, expression declined as pachynema progressed. At the protein level, DNMT3a was the predominant isoform in type B spermatogonia, while DNMT3a2, DNMT3b2, and DNMT3b3 were expressed throughout most of spermatogenesis, except in pachytene spermatocytes. We also detected DNMT3a2 and DNMT3b2 in round spermatids. Taken together, these data highlight the tightly regulated expression of these genes during spermatogenesis and provide evidence that DNMTs may be contributing differentially to the establishment and/or maintenance of methylation patterns in male germ cells.

Keywords: DNA methyltransferase; Dnmt1; Dnmt3a; Dnmt3b; Germ line; DNA methylation; Spermatogenesis; Male germ cells; Epigenetic

Introduction

Methylation of genomic DNA is an epigenetic regulatory mechanism involved in controlling the transcriptional activity of genes and establishing higher order chromatin structures to preserve genome integrity (reviewed by Goll and Bestor, 2004). In mammals, DNA methylation patterns are initially reprogrammed during germ cell development. The patterns differ markedly between male and female gametes, especially at imprinted loci where methylation differences have important implications for allele-specific gene expression in the offspring (reviewed by Reik and Walter, 2001). In the mouse, a major demethylation event takes place in both germ lines between embryonic day (E) 10.5 and E12.5, around the time when primordial germ cells (PGCs) enter the gonads (Hajkova et al., 2002; Kato et al., 1999; Lane et al., 2003; Lee et al., 2002; Szabo and Mann, 1995; Szabo et al., 2002).

Following erasure, DNA methylation patterns are then reestablished in a sex- and sequence-specific manner during gametogenesis. In the male germ line, methylation acquisition begins before birth, in prospermatogonia (Davis et al., 1999, 2000; Kafri et al., 1992; Lees-Murdock et al., 2003; Li et al., 2004; Ueda et al., 2000; Walsh et al., 1998). Between E15 and E19 germ cells begin to stain strongly with an antibody directed against methylated cytosine, indicating the timing of increases in overall global methylation; further methylation changes occur in the few days after birth (Coffigny et al., 1999). At the

* Corresponding author. Montreal Children's Hospital Research Institute, 2300 Tupper Street, Montreal, Quebec, Canada H3H 1P3. Fax: +1 514 412 4331.
E-mail address: jacquetta.trasler@mcgill.ca (J.M. Trasler).

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individual sequence level, the majority of methylated CpGs in the genome are found in repetitive DNA sequences. For repetitive elements such as intracisternal A particles (IAPs), long interspersed nuclear elements (LINEs) and satellite sequences, methylation acquisition is for the most part complete by E17.5 (Lees-Murdock et al., 2003; Walsh et al., 1998). Remethylation of imprinted genes also begins around E15.5, but the process is only completed after birth (Davis et al., 1999, 2000; Li et al., 2004). Developmental studies have shown that the imprinted gene *H19* begins to acquire its methylation marks between E15.5 and E18.5, but only becomes fully methylated postnatally by pachynema (Davis et al., 1999, 2000). Similarly, Chaillet et al. (1991) have demonstrated that an imprinted transgene initially gains its methylation before birth, but the process is only completed postnatally. Assessing the methylation status of a few testis-specific genes has further substantiated methylation acquisition to be continual during spermatogenesis; while some genes are demethylated prior to their expression in the testis, others become de novo methylated (reviewed by Maclean and Wilkinson, 2005). A feature unique to the male germ line is that, in parallel to their establishment, methylation marks must be maintained during DNA replication in spermatogonia and preleptotene spermatocytes; these cells are therefore capable of de novo and maintenance methylation. Conversely in the female germ line, methylation patterns are acquired postnatally during the oocyte growth phase, after the pachytene phase of meiosis is completed and DNA has been replicated (Kono et al., 1996; Lucifero et al., 2002; Walsh et al., 1998).

Both de novo and maintenance DNA (cytosine-5)-methyltransferases (DNMTs) work in concert to create and propagate genomic methylation patterns. Currently, five DNMTs have been characterized and are classified according to similarities found in their C-terminal catalytic domain: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L (reviewed by Goll and Bestor, 2004). Of these, only DNMT1, DNMT3a, and DNMT3b have been proven to have catalytic activity in vivo. DNMT1 is the major methyltransferase in somatic tissues; it has a preference for hemimethylated DNA and is critical for the maintenance of methylation patterns after DNA replication (Bestor, 1992; Li et al., 1992; Lei et al., 1996; Yoder et al., 1997). Sex-specific exons control the expression of *Dnmt1* in the mouse germ line (Mertineit et al., 1998). In the male, DNMT1 is not detected in prenatal gonocytes when methylation patterns are initially laid down, but it is detected in proliferating spermatogonia, as well as leptotene/zygotene spermatocytes (Jue et al., 1995; La Salle et al., 2004; Sakai et al., 2001). Complete downregulation of DNMT1 during pachynema is associated with the expression of Dnmt1p, an untranslated pachytene-specific *Dnmt1* transcript (Jue et al., 1995; Mertineit et al., 1998), whereas DNMT1 becomes reexpressed in round spermatids (Jue et al., 1995; Trasler et al., 1992). It is still unclear which role DNMT1 plays during meiotic prophase or in round spermatids in the absence of DNA replication.

In contrast, DNMT3a and DNMT3b have been postulated to function primarily as de novo DNA methyltransferases (Okano et al., 1998). They are expressed at high levels in mouse embryonic stem (ES) cells and during embryonic development (Okano et al., 1998, 1999; Chen et al., 2003; Watanabe et al., 2002). Expression of *Dnmt3a* is controlled by the use of alternate promoters to produce two different isoforms (Chen et al., 2002). DNMT3a is expressed ubiquitously at low levels and localizes to heterochromatin, suggestive of a housekeeping role. In contrast, DNMT3a2 has been suggested to be more important to de novo methylation because it is expressed at high levels in embryonic stem cells and shows restricted expression in tissues known to undergo de novo methylation such as the testis and the ovary, in addition to localizing to euchromatin. All known isoforms of *Dnmt3b* result from alternative splicing of exons 11, 22, and/or 23 in various combinations (Chen et al., 2002; Ishida et al., 2003; Okano et al., 1998; Weisenberger et al., 2004). Of these, only DNMT3b1 (full-length isoform) and DNMT3b2 (shorter isoform missing the amino acids encoded by exon 11) are capable of DNA methylation (Aoki et al., 2001; Okano et al., 1998). Presumably, the other isoforms are incapable of methylating DNA since their catalytic domain is compromised by splicing of exons 22 and 23; however, they could act as regulators of DNA methylation (Aoki et al., 2001; Chen et al., 2002; Okano et al., 1998; Weisenberger et al., 2004). Interestingly, DNMT3b1 and DNMT3b6 appear to be expressed only in ES cells, while DNMT3b2 and DNMT3b3 are expressed in a restricted manner in somatic tissues (Chen et al., 2002; Weisenberger et al., 2004). We have previously shown that expression of *Dnmt3a* and *Dnmt3b* is highly modulated during testis development (La Salle et al., 2004). Recently, Kaneda et al. (2004) have shown that early germ cell-specific inactivation of *Dnmt3a*, but not *Dnmt3b*, impairs the establishment of de novo methylation patterns in male germ cells, more specifically at paternally imprinted loci, without affecting the methylation status of repeat sequences. Interestingly, inactivation of *Dnmt3L*, a member of the DNMT3 family that lacks DNA methyltransferase activity, produces a similar phenotype: deficient males are infertile, and their germ cells show abnormal DNA methylation acquisition of some repeat elements and imprinted loci (Bourc'his and Bestor, 2004; Bourc'his et al., 2001; Hata et al., 2002; Webster et al., 2005). Studies have started to look at the expression of these genes in germ cells (Lees-Murdock et al., 2005; Sakai et al., 2004; Watanabe et al., 2004), but detailed developmental studies monitoring expression of individual isoforms in key cell types and times when de novo and maintenance methylation are occurring during spermatogenesis are still lacking.

The role each DNA methylating enzyme plays in the genome-wide methylation events that take place throughout male germ cell development, as well as the mechanisms governing their expression, are still unclear. Spermatogenesis is a complex process during which diploid spermatogonia divide and mature into spermatocytes that undergo meiosis to produce haploid spermatids; spermatids go through a specialized maturation process termed spermiogenesis in order to become sperm. Establishment of DNA methylation patterns occurs in spermatogonia and in spermatocytes but is not thought to happen in spermatids. In parallel, maintenance methylation has always been thought to take place in the context of DNA

replication, therefore in mitotically dividing spermatogonia and in preleptotene spermatocytes. Here, we explore how the expression of *Dnmt3a* and *Dnmt3b* is regulated during postnatal spermatogenesis. We report the expression profiles of *Dnmt3a* and *Dnmt3b* in spermatogonia, spermatocytes, and spermatids. We demonstrate that the expression of these genes is highly dynamic in isolated postnatal male germ cells, and we identify windows when the expression of these genes is downregulated. We show that specific germ cell types are marked by the expression of particular *Dnmt3a* transcript variants and isoforms. In addition, we demonstrate that these enzymes are present in spermatids, a cell type not suspected to require de novo or maintenance methylation capacities.

Materials and methods

Mice

Male CD-1 mice were purchased from Charles River Canada Inc. (St. Constant, QC, Canada); the day of birth was designated as postnatal day (dpp) 0. All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee.

Isolation of male germ cells

Purified populations of male germ cells were obtained from the testes of 8-, 17- and 70-dpp mice according to the sedimentation velocity cell separation method (Romrell et al., 1976; Bellve et al., 1977a,b). Briefly, testes of a given age group are collected in RPMI media (Gibco BRL/Invitrogen, Burlington, ON, Canada), enzymatically digested and mechanically disrupted to produce a cell suspension; the cells are then allowed to separate by cellular sedimentation at unit gravity in a 2–4% BSA gradient generated with a STA-PUT apparatus (Johns Scientific, Toronto, ON, Canada). Cells are identified on the basis of morphological criteria and size. Populations of type A spermatogonia (average purity = 86%) and type B spermatogonia (average purity = 83%) were obtained from the testes of 8-dpp mice (n = 2 cell separations). Preleptotene spermatocytes (average purity = 85%), leptotene/zygotene spermatocytes (average purity = 87%) and prepubertal pachytene spermatocytes (average purity = 80%) were obtained from the testes of 17-dpp mice (n = 2 cell separations). Pachytene spermatocytes (average purity = 81%), round spermatids (average purity = 88%), and elongating spermatids mixed with residual bodies (average purity = 86%) were obtained from 70-dpp mice (n = 2 cell separations).

RNA extraction and real-time, quantitative RT-PCR

Total RNA was extracted from snap-frozen pellets of isolated populations of male germ cells using the RNeasy extraction kit with DNaseI treatment and was concentrated using the MinElute kit as described by the manufacturer (Qiagen Inc., Mississauga, ON, Canada). Samples were diluted to 10 ng/μl, dispensed in single-use aliquots and stored at −80°C. Quantitative RT-PCR (QRT-PCR) was performed on the Mx4000 QPCR system from Stratagene (La Jolla, CA) using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Gene-specific primers (La Salle et al., 2004) were used to determine the overall relative expression levels of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* according to the standard curve method (Bustin, 2002). Primers were designed to span introns and pick up all known transcript variants of the various *Dnmt* genes, resulting in the detection of a single band on a gel (data not shown). Transcript variant-specific primers were also designed to determine the relative expression levels of Dnmt3a and Dnmt3a2. Primer sequences were as follows: Dnmt3a forward 5′-CGACCCATGCCAAGACTCACCTTCCAG-3′ (Weisenberger et al., 2002), Dnmt3a2 forward 5′-CCAGACGGGCAGCTATTTAC-3′, and Dnmt3a/3a2 reverse 5′-AGACTCTCCAGAGGCCTGGT-3′; annealing temperatures used were 64°C and 59°C for Dnmt3a and Dnmt3a2, respectively. SYBR Green was used to detect the double-stranded DNA produced during the amplification reaction. Reactions were performed using approximately 10 ng or 100 pg of total RNA for the Dnmts and 18S, respectively. One-step RT-PCR reactions were performed in a 25 μl volume as directed by the manufacturer for 40 cycles. For each product tested, a specific standard curve was established using single-use aliquots of the same stock of RNA (total RNA extracted simultaneously from multiple 6 dpp testes). In all cases, reactions were performed in triplicate on the same two independent sets of germ cells. Specificity was assessed by melting curve analysis and confirmed on a 3% agarose gel after each QRT-PCR experiment (data not shown). QRT-PCR results were normalized to their corresponding 18S rRNA content. Fold changes in expression for a given gene were determined in relation to the expression of that gene in pachytene spermatocytes (calibrator); all other quantities were expressed as an n-fold difference relative to the calibrator. Because the same stock of RNA was used to prepare all standard curves, the relative quantities determined for a given gene using this method could be compared across individual experiments. Representative data for one set of germ cells are presented as mean ± SD.

RT-PCR

The same RNA used for QRT-PCR was used for RT-PCR. RT-PCR reactions were performed using the Qiagen One-step RT-PCR kit as described by the manufacturer. Primers used to discriminate between Dnmt3aα and Dnmt3aβ have been described elsewhere (Weisenberger et al., 2002); primers that span exon 11 and exons 22–23 of the *Dnmt3b* gene were described by Weisenberger et al. (2004). RT-PCR reactions were performed using 20 ng of total RNA in a 25 μl volume. The products were run on a 2% low-melting point agarose gel and stained with ethidium bromide.

Protein extraction and immunoblotting

Proteins were extracted from the testes of 6- and 70-dpp mice or from freshly isolated male germ cells. Protein lysates were prepared by homogenization in 0.15 M NaCl, 0.05 M Tris–Cl (pH 7.4) and 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer's instructions. Total protein aliquots of 70 μg or 7 μg of whole testes or isolated germ cells, respectively, were heated at 65°C for 10 min in reducing sample buffer, electrophoresed on 8% SDS-polyacrylamide gels, and transferred to Hybond ECL nitrocellulose membranes (Amersham, Montreal, QC, Canada). Membranes were blocked in 5% non-fat dried milk and were incubated with one of the following primary antibodies diluted in blocking buffer: clone 64B1446, a monoclonal antibody (mAb) raised against recombinant mouse DNMT3a (1:400; Imgenex, San Diego, CA); NB 100–265, a polyclonal antibody (pAb) raised against amino acids 10–118 of human DNMT3A (1:2000; Novus Biologicals, Littleton, CO); clone 52A1018, a mAb raised against recombinant mouse DNMT3b (1:800; Imgenex); the specificity of these antibodies had previously been assessed by Chen et al. (2002), Beaulieu et al. (2002) and Weisenberger et al. (2004), respectively. Membranes were then washed according to the manufacturer's instruction (Amersham), followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody or a HRP-conjugated goat anti-rabbit IgG antibody (1:7500; Molecular Probes/Invitrogen). After exposure to ECL Plus Western Blotting detection solution (Amersham), chemiluminescence was revealed on Hyperfilm ECL film (Amersham). Coomassie Brilliant Blue staining of gels electrophoresed under identical conditions was used to confirm that equals amounts of proteins were loaded on gel.

Results

Expression of DNA methyltransferases in male germ cells

As DNA methylation pattern establishment and maintenance are ongoing during spermatogenesis, we examined the expression dynamics of *Dnmt3a* and *Dnmt3b* in isolated

postnatal male germ cells using quantitative RT-PCR (QRT-PCR). Germ cells from dissociated testes were fractionated by unit gravity sedimentation on a gradient of BSA as described; eight different populations of male germ cells could be isolated using mice of different age groups. We first determined the overall expression levels of the genes in the various populations of male germ cells purified. Validation of the assay was assessed by determining the expression profile of *Dnmt1*, a DNA methyltransferase that has been well studied in the male germ line (Jue et al., 1995; Trasler et al., 1992). In keeping with previous results obtained by Northern blotting (Jue et al., 1995; Trasler et al., 1992), expression was highest in type A spermatogonia, somewhat lower in type B spermatogonia and preleptotene spermatocytes, and slightly increased in leptotene/zygotene spermatocytes; levels of transcripts decreased as pachynema progressed (Fig. 1A). *Dnmt1* was also expressed in round spermatids at levels comparable to type B spermatogonia but was almost undetectable in residual bodies/elongating spermatids. When global levels of Dnmt3a transcripts were measured, expression results similar to *Dnmt1* were obtained (Fig. 1B). *Dnmt3a* expression was high in type A spermatogonia, slightly decreased in type B spermatogonia and more so in preleptotene spermatocytes, but the decreases were not as marked as the ones observed for *Dnmt1* in the same germ cell types. Expression in leptotene/zygotene spermatocytes was about the same as in type A spermatogonia, only to decrease through pachynema. Again, Dnmt3a transcripts were detected in round spermatids but were at their lowest level in residual bodies/elongating spermatids. In contrast, *Dnmt3b* expression was a lot more dynamic during male germ cell development, increases and decreases in expression being more pronounced for this gene (Fig. 1C). *Dnmt3b* expression was highest in type A spermatogonia, decreased dramatically in type B spermatogonia and preleptotene spermatocytes (by more than 4-fold), only to increase again by almost 2-fold in leptotene/zygotene spermatocytes. Once more, progression through pachynema was associated with a steady decrease in expression, while Dnmt3b transcripts were clearly detected in round spermatids. Dnmt3a and Dnmt3b transcripts can therefore be detected at all stages of male germ cell development tested, even at stages when de novo and maintenance methylation have not been shown to take place (see Supplementary Fig. 7 for a comparison of the relative expression levels of Dnmt3a, Dnmt3b, and Dnmt1 transcripts).

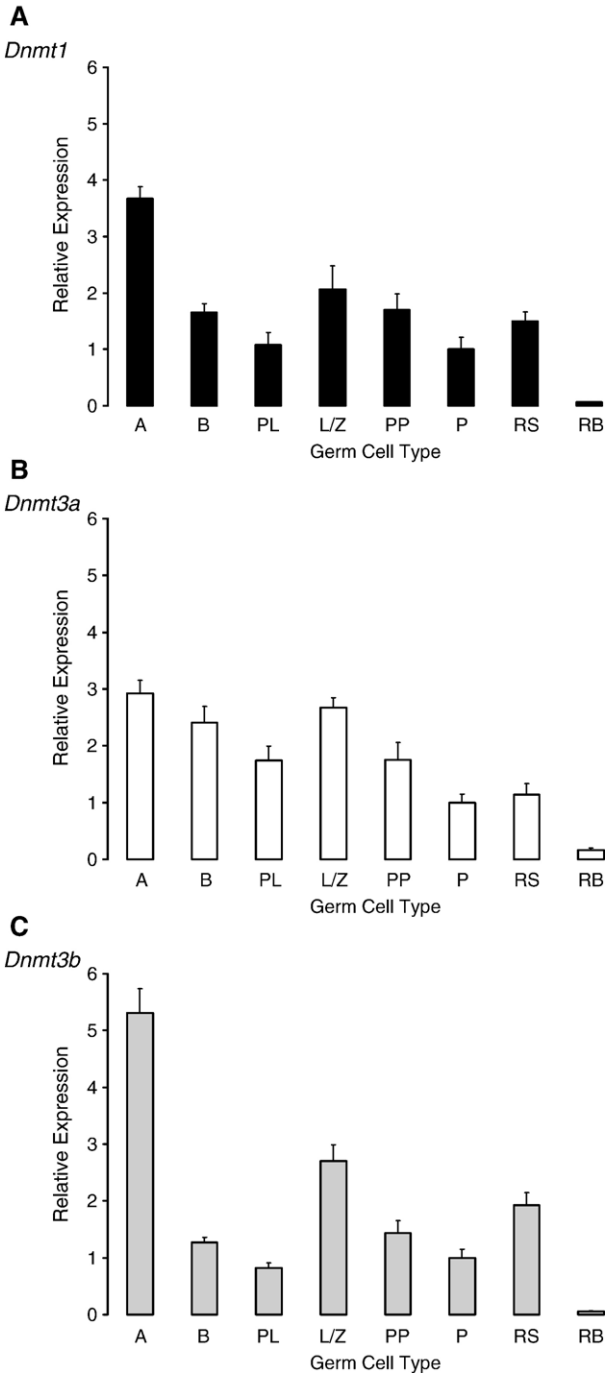

Fig. 1. Expression dynamics of DNA methyltransferases during male germ cell development. Relative quantification of (A) *Dnmt1*, (B) *Dnmt3a*, and (C) *Dnmt3b* expression in isolated populations of male germ cells. Real-time RT-PCR was used to determine the expression levels of the three *Dnmt* genes in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/elongating spermatids (RB). Expression of each gene was determined in triplicate in each of the two series of germ cells; shown here are the mean expression results obtained for one series. Mean ± SD.

Transcriptional control of Dnmt3a expression: usage of alternative first exons and promoters

Transcriptional regulation at the *Dnmt3a* locus is multifaceted (Figs. 2A–3A). We used QRT-PCR to tease out the discrepancies in expression between Dnmt3a and Dnmt3a2, the transcripts resulting in the production of the two main protein products (Fig. 2B). A forward primer unique to either Dnmt3a or Dnmt3a2 was used in combination with a reverse primer common to both products; the individual PCR products were amplified in separate reactions due to the type of detection method employed. Two distinct expression profiles were obtained for Dnmt3a and Dnmt3a2. Dnmt3a (Fig. 2B, top panel) was expressed relatively constantly until pachynema, even if its expression did seem to increase in type B

spermatogonia, and was not significantly expressed in spermatids. Expression in spermatids and residual bodies could not be determined because the initial amount of Dnmt3a template was not sufficient in these samples to be distinguished above background and produce a Ct (threshold cycle) value that could be analyzed. Dnmt3a2 (Fig. 2B, bottom panel) presented a very dynamic expression pattern reminiscent of the one obtained for *Dnmt3b* (Fig. 1C). Expression of Dnmt3a2 was highest in type A spermatogonia, drastically decreased in type B spermatogonia and preleptotene spermatocytes, and increased again in leptotene/zygotene spermatocytes to levels almost as high as in type A spermatogonia. There was an abrupt drop in expression in prepubertal pachytene spermatocytes that persisted in more mature pachytene spermatocytes. Contrary to Dnmt3a, Dnmt3a2 could be amplified in round spermatids and to a lower extent in residual bodies/elongating spermatids. Clear differences in expression could therefore be detected between Dnmt3a and Dnmt3a2, suggesting that their two protein products could be playing distinct roles during male germ cell development (see Supplementary Fig. 8 for a comparison of the relative expression levels of Dnmt3a and Dnmt3a2 transcripts).

Additional alternatively spliced variants of *Dnmt3a* have also been described and are illustrated in Fig. 3A (Weisenberger et al., 2002). Dnmt3a can be produced from the use of alternate 5′ first exons (exons 1α and 1β) and transcripts that include intron 4 have also been detected. Transcripts

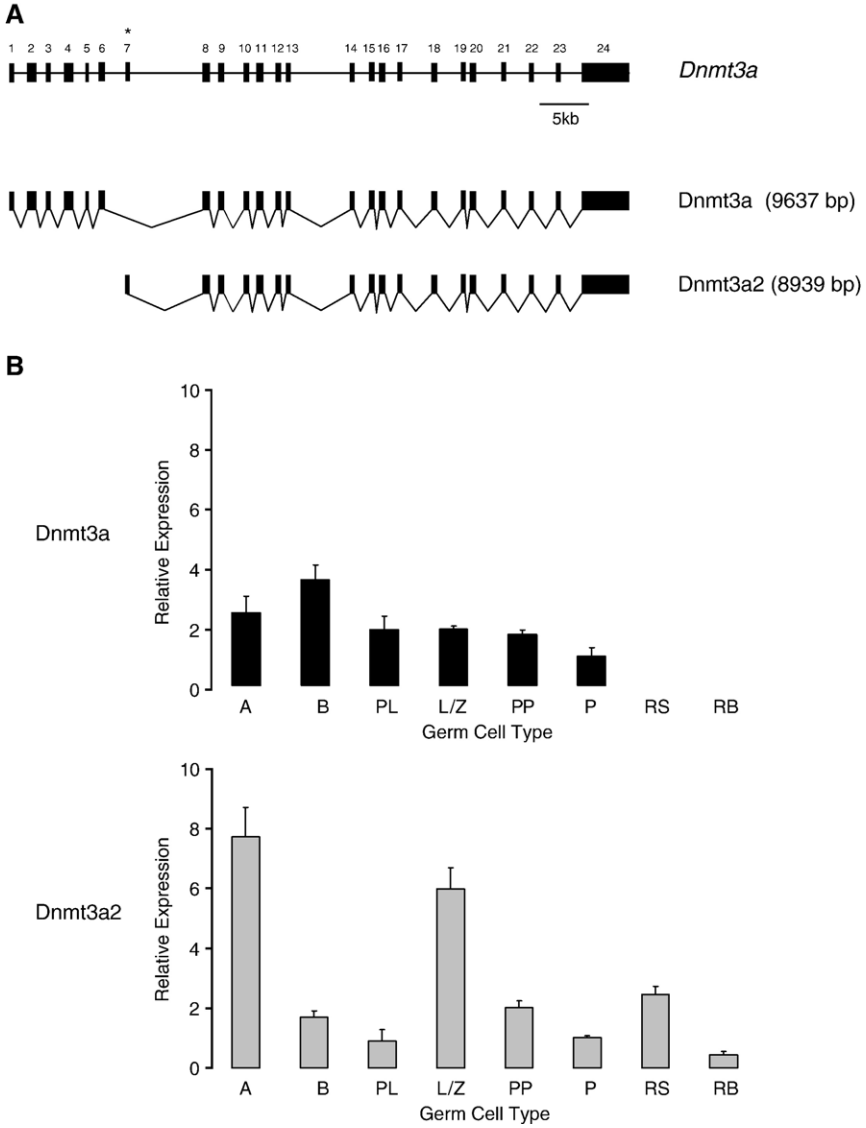

Fig. 2. Differential expression of Dnmt3a and Dnmt3a2 in male germ cells. (A) Structure of the mouse *Dnmt3a* locus (top) and the two main mRNA species produced from it (bottom). The Dnmt3a2-unique exon is marked by an asterisk. Exons are shown as black bars (exon sizes and positions are not to scale; adapted from Chen et al., 2002). (B) Relative expression of Dnmt3a (top) and Dnmt3a2 (bottom) in purified populations of male germ cells. QRT-PCR was used to determine the expression levels of the two transcripts in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/elongating spermatids (RB). Expression of each transcript was determined in triplicate in each of the two series of germ cells; shown here are the mean expression results obtained for one series. Mean ± SD.

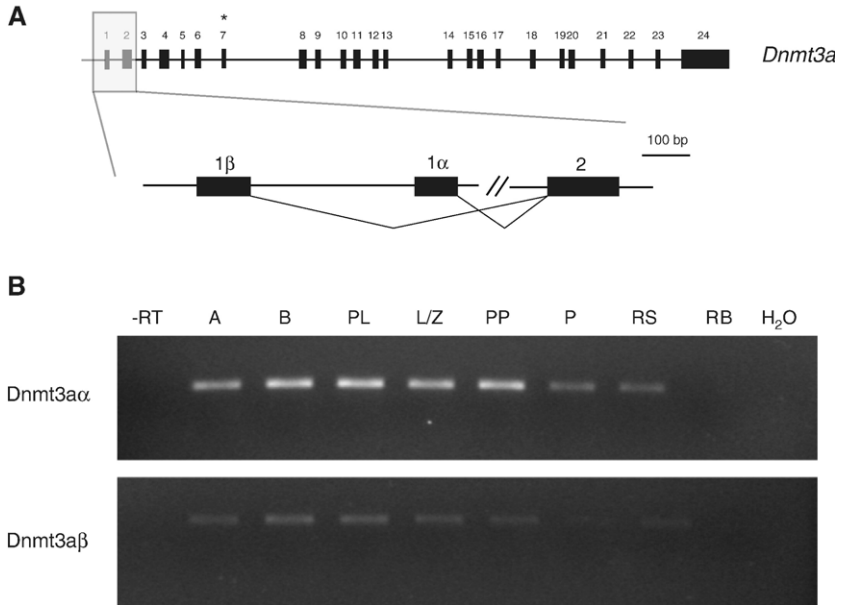

Fig. 3. Expression of Dnmt3aα and *Dnmt3a*β in mouse germ cells. (A) Schematic representation of the 5′ region of the mouse *Dnmt3a* gene. The shaded area enlarged below shows an additional first exon (exon 1β) situated 5′ of the first exon that was initially identified (exon 1α). Splicing of exon 1α to exon 2 produces the Dnmt3aα transcript, while splicing of exon 1β to exon 2 gives rise to the Dnmt3aβ transcript (both splicing events are depicted here). Exons are represented as black bars (adapted from Weisenberger et al., 2002). (B) Expression of both Dnmt3aα and Dnmt3aβ in isolated male germ cells. RT-PCR was used to establish the incidence of transcription through exons 1α and 1β in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/elongating spermatids (RB). −RT, negative control lacking reverse transcriptase.

containing exon 1α (Dnmt3aα) are usually detected in somatic cells, whereas exon1β-containing transcripts (Dnmt3aβ) are preferentially expressed in mouse ES cells (Weisenberger et al., 2002). Having monitored the levels of Dnmt3a transcripts by QRT-PCR without being able to discriminate between Dnmt3aα and Dnmt3aβ, we wanted to determine if transcription went through both first exons in male germ cells. Contribution of the two first exons was evaluated using RT-PCR and was found to proceed through both first exons (Fig. 3B). Interestingly, we were able to detect Dnmt3a transcripts in round spermatids using this approach, albeit at very low levels, conversely to when QRT-PCR was used. We next tried to detect the presence of intron 4-containing transcripts using a similar RT-PCR strategy but were unable to detect any of these transcripts in male germ cells (data not shown). Overall, the data indicate that Dnmt3aα, Dnmt3aβ, and Dnmt3a2 are the main forms of *Dnmt3a* transcripts present in male germ cells and suggest that tight control of transcription takes place by use of different mechanisms (alternate promoter or first exon) throughout spermatogenesis.

Production of multiple Dnmt3b transcripts by alternative splicing in male germ cells

The mouse *Dnmt3b* gene contains 24 exons, of which exons 11, 22, and/or 23 can be alternatively spliced to produce eight different transcripts (Fig. 4A; Ishida et al., 2003; Weisenberger et al., 2004). Weisenberger et al. (2004) determined that exon 11-spliced transcripts are usually expressed in somatic cells, whereas unspliced products are mostly expressed in ES cells. Using primers that span the two splicing events, we established the occurrence of these events in male germ cells by means of RT-PCR. We could see that transcripts with a spliced exon 11 were usually more prevalent (Fig. 4B, top panel), and that at least three of the four possible splicing combinations at exons 22 and/or 23 (which affect the integrity of the catalytic domain) could be detected at all stages of male germ cell development tested (Fig. 4B, bottom panel). Since spliced-exon 11 mRNA species are more abundant, and that the strongest amplification was obtained for intact exons 22 and 23, we can predict that the predominant form of *Dnmt3b* mRNA is Dnmt3b2.

Immunoblot analysis of DNMT3a and DNMT3b expression during male gametogenesis

Having established the expression profiles of *Dnmt3a* and *Dnmt3b* at the mRNA level during male germ cell development, we undertook a similar analysis of protein expression. Lysates of purified germ cells were analyzed by immunoblot using commercially available antibodies. For *Dnmt3a*, two antibodies were used to detect the presence of DNMT3a and DNMT3a2 (see Fig. 5A for epitope mapping). Consistent with its epitope mapping to the N-terminus, antibody NB 100–265 detected a single band of ~130 kDa corresponding to DNMT3a only (Fig. 5B, top panel). DNMT3a was detected in type A and B spermatogonia, preleptotene, leptotene/zygotene, and prepubertal pachytene spermatocytes but was completely absent in pachytene spermatocytes. Most surprisingly, DNMT3a

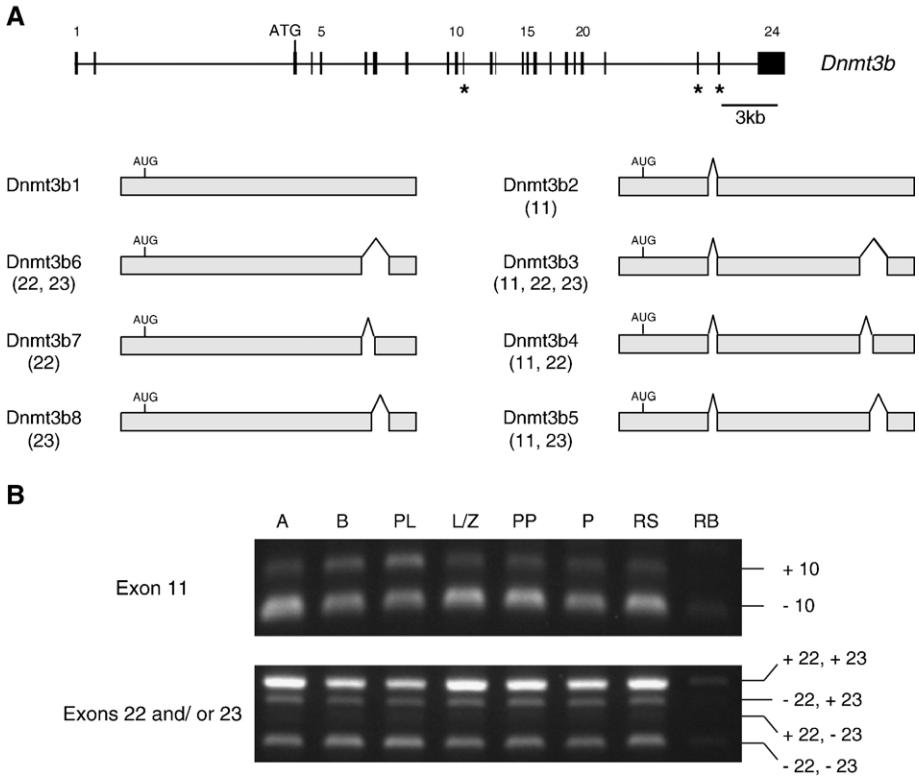

Fig. 4. Expression of multiple Dnmt3b transcripts in male germ cells. (A) Genomic organization of the mouse *Dnmt3b* gene. (Top) Structural organization of the gene. Exons that can be spliced are marked by asterisks. Exons are shown by filled boxes; exon size and position are to scale. ATG, the translation initiation codon. (Bottom) Representation of the eight possible mRNA species created by alternative splicing of exons 11, 22, and 23. Transcripts are depicted as grey boxes with open areas where splicing occurs. Spliced exons are shown in parentheses. (Adapted from Ishida et al., 2003; Weisenberger et al., 2004). (B) Analysis of the occurrence of the two alternative splicing events: exon 11 and exons 22 and/or 23. Prevalence of the splicing events was determined by RT-PCR in type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/elongating spermatids (RB). + or −, unspliced or spliced exon, respectively.

expression peaked in type B spermatogonia, consistent with the mRNA expression profile; it was not detected in either round spermatids or in residual bodies/elongating spermatids. When mAb 64B1446 was used, an additional band of ~100 kDa corresponding to DNMT3a2 was detected in addition to the 130-kDa band corresponding to DNMT3a (Fig. 5B, middle panel). Albeit the bands were fainter most likely due to antibody titer, a profile similar to the one obtained with pAb NB 100–265 was detected for DNMT3a, expression peaking again in type B spermatogonia. The profile obtained for the 100-kDa band was very different: DNMT3a2 expression was highest in type A spermatogonia and leptotene/zygotene spermatocytes, was present at lower levels in type B spermatogonia and preleptotene spermatocytes, but decreased in prepubertal pachytene spermatocytes and became almost absent in pachytene spermatocytes. DNMT3a2 was then reexpressed in round spermatids but was not detected in residual bodies/elongating spermatids. Using a combination of antibodies, we were able to show distinctive expression profiles for DNMT3a and DNMT3a2 in germ cells.

For *Dnmt3b*, only one antibody capable of detecting all isoforms was used (Weisenberger et al., 2004). The RT-PCR data presented here as well as a study done by Chen et al. (2002) on adult tissues including the testis suggested that Dnmt3b2 and Dnmt3b3 were the two major transcripts of the male germ line. Because DNMT3b isoforms have similar molecular weights, we compared the migration profiles obtained for the germ cell lysates to the one obtained for 6 and 70 dpp testis protein extracts (Fig. 5C). We could detect five bands in the 6 dpp extract, two bands being more prominent than the others. The highest molecular weight band was consistent with the weight expected for DNMT3b1 (~120 kDa) and is only very faintly expressed. The following, predominant two bands most likely corresponded to DNMT3b2 and DNMT3b3, since the most abundant mRNA species detected in the adult testis and in germ cells were ones containing and lacking exons 22/23, respectively (Fig. 4B; Chen et al., 2002). The last two bands were of much smaller molecular weight and could correspond to DNMT3b4 or DNMT3b7, since transcripts lacking exon 22 were also detected in germ cells (Fig. 4B). We could only detect 3 bands in the 70 dpp extract, DNMT3b2 and DNMT3b3 most likely being the two predominant isoforms as Chen et al. (2002) had suggested. When we looked at the migration profile obtained for germ cell lysates, we saw only two predominant bands that migrate at the same rate as the bands corresponding to DNMT3b2 and DNMT3b3 in the 6 and 70 dpp testis lanes (Fig. 5C). According to the RT-PCR results (Fig. 4B), other isoforms could be present in germ cells, but they are either expressed at levels too low to be

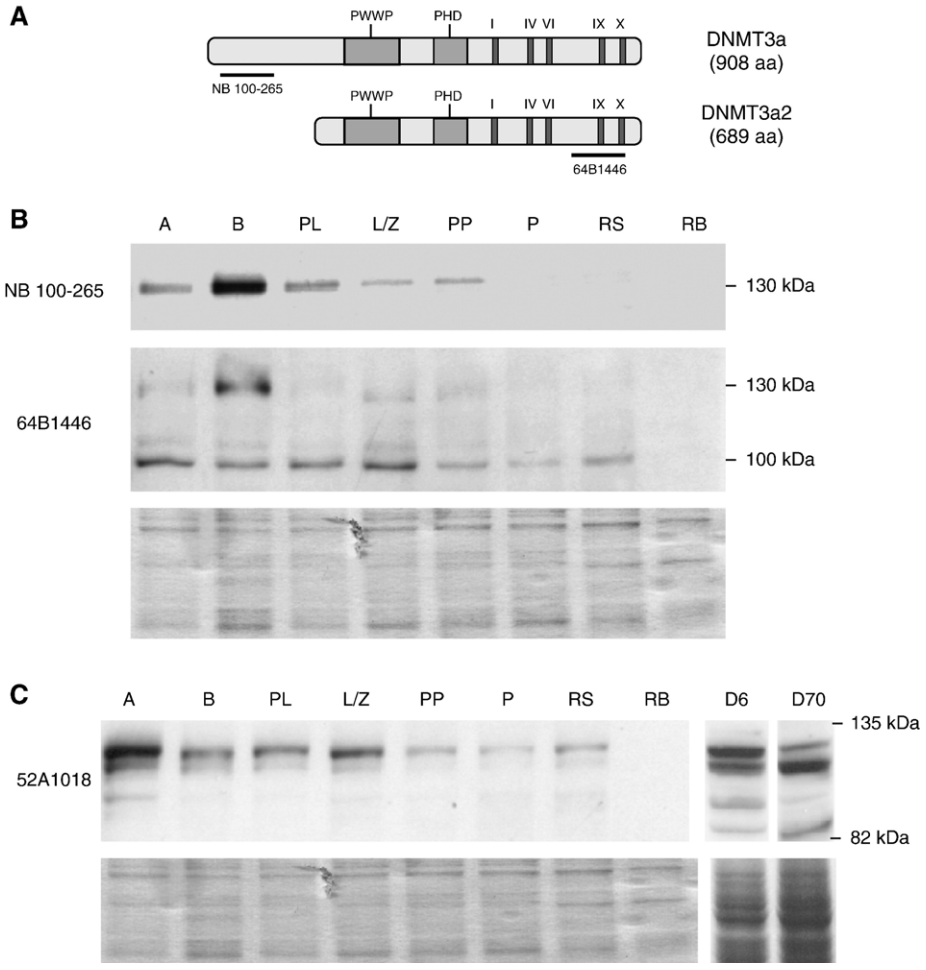

Fig. 5. Immunoblot detection of DNMT3a and DNMT3b in purified populations of male germ cells. (A) Schematic representation of DNMT3a and DNMT3a2. The conserved PWWP and PHD domains and methyltransferase motifs (I, IV, VI, IX, X) are depicted. The location of the different epitopes is underlined. (B) Expression of DNMT3a and DNMT3a2 in male germ cells. Top panel: detection of DNMT3a in protein lysates obtained from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/elongating spermatids (RB) using the NB 100–265 antibody. Middle panel: expression of DNMT3a and DNMT3a2 in the same germ cell populations using the 64B1446 antibody. Bottom panel: gel electrophoresed under identical conditions but stained with Coomassie Brilliant blue dye demonstrating equal protein loading in each lane. (C) Detection of multiple DNMT3b isoforms in male germ cells. Upper panel: Western blot showing the presence of several DNMT3b isoforms in total protein extracted from various populations of male germ cells and from 6 (D6)- and 70 (D70)-dpp testes using the 52A1018 antibody (lane abbreviations are the same as in B for germ cells). Lower panel: gel electrophoresed under identical conditions but stained with Coomassie Brilliant blue dye demonstrating equal protein loading in each lane.

detected in these conditions or these mRNA remain untranslated.

Discussion

DNA methylation events taking place during spermatogenesis have important implications for gamete integrity and transmission of epigenetic information to the next generation (Bourc'his and Bestor, 2004; Kaneda et al., 2004). Recent studies have started to shed light on DNA methyltransferases that might be involved in these important processes (Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004), but it is still unclear if all DNMTs contribute equally to the establishment and the maintenance of DNA methylation patterns throughout spermatogenesis. We provide evidence that the expression of the genes encoding the postulated de novo DNA methyltransferases is developmentally regulated during male germ cell development. We show that proliferating and differentiating male germ cells are marked by distinctive Dnmt3a and Dnmt3b expression profiles and that specific transcript variants as well as isoforms are expressed in particular cell types. We also demonstrate that *Dnmt3a* and *Dnmt3b* expression is down-regulated both at the RNA and protein levels during pachynema, as it is the case for *Dnmt1*. Finally, we clearly show that DNMT3a and DNMT3b isoforms are detected in round spermatids.

There is growing evidence that DNMT family members directly interact and cooperate to establish and maintain DNA methylation patterns (Chen et al., 2003; Datta et al., 2003; Hata et al., 2002; Kim et al., 2002; Margot et al., 2003). Since DNMTs do not appear to have any sequence specificity beyond

CpG dinucleotides, multiple mechanisms are proposed to explain how DNA methyltransferases can find their targets in the genome, including differential accessibility to chromosomal regions and recruitment of DNMTs to specific sequences by accessory factors (reviewed by Li, 2002). Regulation of the establishment of DNA methylation patterns has been suggested to be controlled by differential expression of *Dnmt3a* and *Dnmt3b* isoforms by Chen et al. (2003); different enzymes would have both common and preferred target sequences. In their system, minor satellite repeats were methylated by DNMT3b1, major satellite repeats were methylated by DNMT3a and DNMT3a2, while the 5′ region of *H19* was only methylated by DNMT3a2. Consistent with that, gene-targeting studies clearly demonstrate that not all sequences are affected equally upon *Dnmt* inactivation (Bourc'his and Bestor, 2004; Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004; Li et al., 1992; Okano et al., 1999; Webster et al., 2005). DNMT3b-deficient mice show demethylation of minor satellite repeats, while inactivation of *Dnmt3L* leads to incomplete acquisition of methylation at the paternally imprinted loci *H19* and *Rasgrf1*, in addition to preventing methylation of LINE-1 and IAP repeat elements in the male germ line (Bourc'his and Bestor, 2004; Okano et al., 1999; Webster et al., 2005). Of particular interest is a recent report by Kaneda et al. (2004) showing that acquisition of DNA methylation at some but not all imprinted loci is impaired when *Dnmt3a* is specifically inactivated in germ cells. Preference for different genomic sequences may simply reflect differences in chromatin accessibility, given that DNMT3a and DNMT3b localize to different subcellular compartments (Bachman et al., 2001; Chen et al., 2002). Nonetheless, how sequence-specific methylation patterns are generated during gametogenesis escapes our understanding.

In the male germ line, acquisition of methylation begins prenatally in gonocytes and is further consolidated following birth until pachynema. In parallel, acquired marks have to be maintained as DNA is replicated in spermatogonia and preleptotene spermatocytes. We have previously demonstrated that expression of the *Dnmt* genes is highly dynamic during testis development and proposed that DNMT3a and DNMT3L might interact together to establish DNA methylation patterns in prenatal gonocytes, while DNMT3b and DNMT1 could be cooperating to maintain genome integrity after birth (La Salle et al., 2004). Here, we further investigate these findings by exploring the expression pattern of the active de novo DNA methyltransferases in isolated postnatal male germ cells (summarized in Fig. 6). Several lines of evidence indicate that the use of isolated germ cells along with QRT-PCR is a reasonable approach for studying the expression of the different *Dnmt* genes during postnatal spermatogenesis. In our previous studies, *Dnmt1* expression patterns were examined using Northern and Western blotting and immunocytochemistry on isolated cells as well as immunocytochemistry on testicular tissue (Jue et al., 1995; Mertineit et al., 1998). Here, the *Dnmt1* QRT-PCR profile fits closely with our previously published DNMT1 protein expression and immunocytochemistry data. In the current study, the *Dnmt3a* and *Dnmt3b* QRT-PCR germ cell expression data were similar to the protein expression profiles. Furthermore, in keeping with our results, Watanabe et al. (2004) used immunocytochemistry on early postnatal testicular tissue and reported expression of DNMT3a in type B spermatogonia and preleptotene spermatocytes and DNMT3b in type A spermatogonia.

In the current study, when expression of *Dnmt3a* and *Dnmt3b* is probed and compared to that of *Dnmt1*, a similar pattern of expression is identified for all three genes albeit with small differences. Dnmt1 and Dnmt3b expression is highest in type A spermatogonia but diminished in type B spermatogonia and preleptotene spermatocytes; Dnmt3a is expressed similarly but expression is not reduced as extensively. Progression through meiosis is also associated with a decrease in expression of all three genes, but expression is turned on again in round spermatids. Based on these results, we identify two developmental windows during which transcription of these genes has to be downregulated during spermatogenesis: (1) differentiation of spermatogonia into spermatocytes and (2) pachynema. These data are the first indication of how tightly regulated the expression of *Dnmt3a* and *Dnmt3b* is during spermatogenesis. We postulate that the down regulation of the DNMTs at two times during spermatogenesis could be related either to the need to express testis-specific genes or to changes in chromatin structure. Although there are a number of examples of testis-specific genes that show a correlation between hypomethylation and germ cell expression, more studies are needed, and it is presently unresolved whether alterations in methylation are a cause or consequence of events associated with gene transcription (for review, see Maclean and Wilkinson, 2005). At the chromatin level, distinct differences in core histone acetylation have been found in spermatogonia and spermatocytes (Hazzouri et al., 2000) and Coffigny et al. (1999) have hypothesized that genome-wide changes in 5-methyl cytosine staining in germ cells in the perinatal testis might be associated with functional genomic reorganization related to cellular differentiation.

Assessing the mechanisms that control the transcriptional activity of *Dnmt3a* provides further support for the tight regulation of the *Dnmt3s* during spermatogenesis. Alternate promoter usage at the *Dnmt3a* locus gives rise to two transcripts: Dnmt3a and Dnmt3a2. Evaluating the relative amounts of these transcripts in male germ cells exposed, surprisingly, two distinct expression profiles. While the profile obtained for Dnmt3a2 resembles that of Dnmt1 and Dnmt3b, the one obtained for Dnmt3a is strikingly different. Instead of being downregulated in type B spermatogonia and preleptotene spermatocytes, expression of Dnmt3a increases in these cells. These data differ markedly from what Lees-Murdock et al. (2005) found, where they did not detect any significant difference in expression between Dnmt3a and Dnmt3a2 in the testis. Because non-quantitative RT-PCR was conducted on whole gonads at only three times during development, expression differences would most likely not have been detected. Since the ratio of somatic cells to germ cells as well as the proportion of different germ cells types change drastically during the first wave of spermatogenesis, expression at multiple times during testis development or in

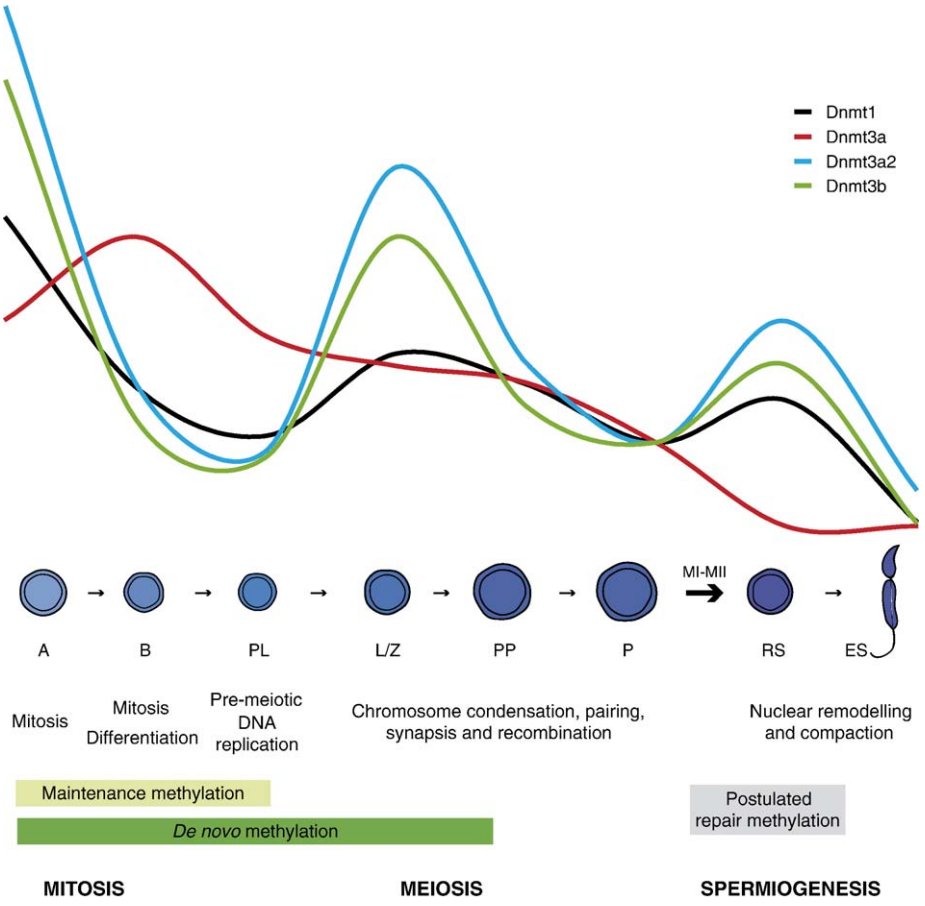

Fig. 6. Schematic representation of DNA methyltransferase dynamics during spermatogenesis. Progression of methylation pattern establishment and maintenance, and expression of Dnmt1 (black), Dnmt3a (red), Dnmt3a2 (blue) and Dnmt3b (green) are presented as functions of male germ cell development. The intensity of the blue shading in germ cells reflects the methylation status of the paternal genome. Some of the most characteristic biological processes associated with mitosis, meiosis, and spermiogenesis are also depicted. Representative cell types are indicated as follows: A, type A spermatogonia; B, type B spermatogonia; PL, preleptotene spermatocyte, L/Z, leptotene/zygotene spermatocyte; PP, prepubertal pachytene spermatocyte; P, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatids. MI–MII, meiosis I–II.

isolated populations of male germ cells has to be considered. Immunoblotting analyses confirm the patterns we find for Dnmt3a and Dnmt3a2 at the protein level and clearly demonstrate DNMT3a to be the major *Dnmt3a* isoform in type B spermatogonia. Consistent with our findings is an immunofluorescence study conducted by Watanabe et al. (2004) on adult testis sections, where another antibody that recognizes DNMT3a detected this isoform only at stages where type B spermatogonia and preleptotene spermatocytes are found; however, their antibody did not allow them to examine DNMT3a2 expression. Our analyses also reveal that both DNMT3a and DNMT3a2 are absent in pachytene spermatocytes, but that DNMT3a2 is present in round spermatids, results reminiscent of DNMT1 (Jue et al., 1995; Trasler et al., 1992).

A different regulatory mechanism, namely alternative splicing, gives rise to the DNMT3b isoforms. While DNMT3b1 and DNMT3b2 are postulated to function as de novo DNA methyltransferases, the role of the other isoforms beyond being hypothetical regulators of DNA methylation remains elusive. Although Kaneda et al. (2004) have suggested that *Dnmt3b* might not be required for spermatogenesis, a role for this enzyme in the male germ line cannot be excluded since the details concerning this conditional knock-out model have not been reported yet. Compensatory mechanisms could be activated in the absence of DNMT3b, attenuating the appearance of a phenotype. We find that DNMT3b1 is unlikely to be involved in de novo methylation in the male germ line, as the corresponding transcript is presumably not expressed since unspliced-exon 11 transcripts are rare, and we are unable to detect the protein by immunoblotting. Our data show that the main transcript detected in male germ cells that can produce a catalytically active protein and carry out de novo methylation is Dnmt3b2. Consistent with these results, we detect DNMT3b2 by immunoblotting at high levels in type A spermatogonia and in leptotene/zygotene spermatocytes, cell types where de novo methylation is believed to take place. Previous studies have also shown that transcripts lacking exon 22 and exons 22/23 can be amplified at different stages of postnatal testis development (Chen et al., 2002; Lees-Murdock et al., 2005). However, because whole gonads were used, it was impossible to conclude if these transcripts are specifically expressed in germ cells. Here, we show that transcripts lacking exon 22 and exons 22/23 are present in isolated postnatal male

germ cells; however, only the DNMT3b3 isoform can be detected in these same germ cells. Taken together, these results suggest that DNMT3b2 and DNMT3b3 are the two major *Dnmt3b* isoforms in male germ cells. DNMT3b3 could serve as a positive regulator of DNMT3b2 or of another DNMT similarly to DNMT3L, or it could target DNMTs to loci destined to be methylated (Chedin et al., 2002; Weisenberger et al., 2004). On the flip side, DNMT3b3 could act as a dominant negative regulator of DNA methylation by competing for target sites (Saito et al., 2002).

Interestingly, DNMT3a and DNMT3b, like DNMT1, are reexpressed in round spermatids. One role for DNMTs late in spermatogenesis could be for the remethylation of testis-specific genes. For instance, phosphoglycerate kinase-2 is remethylated during transit through the epididymis (Ariel et al., 1994; Geyer et al., 2004). Another possible role for DNMTs during spermiogenesis is for DNA repair, more specifically for the restoration of epigenetic information. A recent report by Mortusewicz et al. (2005) shows that in somatic cells, DNMT1 is recruited to sites of DNA damage induced by microirradiation, suggesting a role for DNMTs in repair.

Several interesting features pertaining to DNMT1 in the germ line have been exposed during the last decade, including highly regulated expression during gametogenesis and the use of sex-specific exons to control the production of translated and non-translated RNAs. Nonetheless, issues related to the role of DNMT1 in the male germ line during meiosis and in round spermatids, as well as the relevance of its down-regulation during pachynema, remain unresolved. The findings presented here further support a common regulatory theme for the expression of DNA methyltransferases during key periods of spermatogenesis. Our data clearly demonstrate that all of the DNMT3a and DNMT3b isoforms are downregulated during pachynema, emphasizing the need to restrict expression of DNMTs during this period of male germ cell development. We also show that DNMT3a is the predominant DNMT in differentiating spermatogonia, suggestive of a specific role for this isoform in these cells. In light of our understanding of DNA methylation patterning in the male germ line, our data strongly indicate that DNMT3a2, DNMT3b2, and DNMT3b3 could be involved in de novo methylation in spermatogonia and spermatocytes, in addition to repair-associated de novo or maintenance DNA methylation in round spermatids. In conclusion, the data presented here set the stage for in-depth gene-targeting studies by emphasizing the need to inactivate specific transcript variants or isoforms of the DNA methyltransferase genes at different times during male germ cell development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.436.

References

Aoki, A., Suetake, I., Miyagawa, J., Fujio, T., Chijiwa, T., Sasaki, H., Tajima, S., 2001. Enzymatic properties of de novo-type mouse DNA (cytosine-5) methyltransferases. Nucleic Acids Res. 29, 3506–3512.

Ariel, M., Cedar, H., McCarrey, J., 1994. Developmental changes in methylation of spermatogenesis-specific genes include reprogramming in the epididymis. Nat. Genet. 7, 59–63.

Bachman, K.E., Rountree, M.R., Baylin, S.B., 2001. Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. J. Biol. Chem. 276, 32282–32287.

Beaulieu, N., Morin, S., Chute, I.C., Robert, M.F., Nguyen, H., MacLeod, A.R., 2002. An essential role for DNA methyltransferase DNMT3B in cancer cell survival. J. Biol. Chem. 277, 28176–28181.

Bellve, A.R., Cavicchia, J.C., Millette, C.F., O'Brien, D.A., Bhatnagar, Y.M., Dym, M., 1977a. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J. Cell Biol. 74, 68–85.

Bellve, A.R., Millette, C.F., Bhatnagar, Y.M., O'Brien, D.A., 1977b. Dissociation of the mouse testis and characterization of isolated spermatogenic cells. J. Histochem. Cytochem. 25, 480–494.

Bestor, T.H., 1992. Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. EMBO J. 11, 2611–2617.

Bourc'his, D., Bestor, T.H., 2004. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature 431, 96–99.

Bourc'his, D., Xu, G.L., Lin, C.S., Bollman, B., Bestor, T.H., 2001. Dnmt3L and the establishment of maternal genomic imprints. Science 294, 2536–2539.

Bustin, S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29, 23–39.

Chaillet, J.R., Vogt, T.F., Beier, D.R., Leder, P., 1991. Parental-specific methylation of an imprinted transgene is established during gametogenesis and progressively changes during embryogenesis. Cell 66, 77–83.

Chedin, F., Lieber, M.R., Hsieh, C.L., 2002. The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. Proc. Natl. Acad. Sci. U. S. A. 99, 16916–16921.

Chen, T., Ueda, Y., Xie, S., Li, E., 2002. A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. J. Biol. Chem. 277, 38746–38754.

Chen, T., Ueda, Y., Dodge, J.E., Wang, Z., Li, E., 2003. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. Mol. Cell. Biol. 23, 5594–5605.

Coffigny, H., Bourgeois, C., Ricoul, M., Bernardino, J., Vilain, A., Niveleau, A., Malfoy, B., Dutrillaux, B., 1999. Alterations of DNA methylation patterns in germ cells and Sertoli cells from developing mouse testis. Cytogenet. Cell Genet. 87, 175–181.

Datta, J., Ghoshal, K., Sharma, S.M., Tajima, S., Jacob, S.T., 2003. Biochemical fractionation reveals association of DNA methyltransferase (Dnmt) 3b with Dnmt1 and that of Dnmt 3a with a histone H3 methyltransferase and Hdac1. J. Cell. Biochem. 88, 855–864.

Davis, T.L., Trasler, J.M., Moss, S.B., Yang, G.J., Bartolomei, M.S., 1999. Acquisition of the *H19* methylation imprint occurs differentially on the parental alleles during spermatogenesis. Genomics 58, 18–28.

Davis, T.L., Yang, G.J., McCarrey, J.R., Bartolomei, M.S., 2000. The *H19* methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. Hum. Mol. Genet. 9, 2885–2894.

Geyer, C.B., Kiefer, C.M., Yang, T.P., McCarrey, J.R., 2004. Ontogeny of a demethylation domain and its relationship to activation of tissue-specific transcription. Biol. Reprod. 71, 837–844.

Goll, M.G., Bestor, T.H., 2004. Eukaryotic cytosine methyltransferases. Annu. Rev. Biochem. 74, 481–514.

Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El Maarri, O., Reik, W., Walter, J., Surani, M.A., 2002. Epigenetic reprogramming in mouse primordial germ cells. Mech. Dev. 117, 15–23.

Hata, K., Okano, M., Lei, H., Li, E., 2002. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development 129, 1983–1993.

Hazzouri, M., Pivot-Pajot, C., Faure, A.K., Usson, Y., Pelletier, R., Sele, B., Khochbin, S., Rousseaux, S., 2000. Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone deacetylases. Eur. J. Cell Biol. 79, 950–960.

Ishida, C., Ura, K., Hirao, A., Sasaki, H., Toyoda, A., Sakaki, Y., Niwa, H., Li, E., Kaneda, Y., 2003. Genomic organization and promoter analysis of the Dnmt3b gene. Gene 310, 151–159.

Jue, K., Bestor, T.H., Trasler, J.M., 1995. Regulated synthesis and localization of DNA methyltransferase during spermatogenesis. Biol. Reprod. 53, 561–569.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H., Razin, A., 1992. Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev. 6, 705–714.

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., Sasaki, H., 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 429, 900–903.

Kato, Y., Rideout III, W.M., Hilton, K., Barton, S.C., Tsunoda, Y., Surani, M.A., 1999. Developmental potential of mouse primordial germ cells. Development 126, 1823–1832.

Kim, G.D., Ni, J., Kelesoglu, N., Roberts, R.J., Pradhan, S., 2002. Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. EMBO J. 21, 4183–4195.

Kono, T., Obata, Y., Yoshimzu, T., Nakahara, T., Carroll, J., 1996. Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. Nat. Genet. 13, 91–94.

La Salle, S., Mertineit, C., Taketo, T., Moens, P.B., Bestor, T.H., Trasler, J.M., 2004. Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells. Dev. Biol. 268, 403–415.

Lane, N., Dean, W., Erhardt, S., Hajkova, P., Surani, A., Walter, J., Reik, W., 2003. Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis 35, 88–93.

Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A., Ishino, F., 2002. Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development 129, 1807–1817.

Lees-Murdock, D.J., De Felici, M., Walsh, C.P., 2003. Methylation dynamics of repetitive DNA elements in the mouse germ cell lineage. Genomics 82, 230–237.

Lees-Murdock, D.J., Shovlin, T.C., Gardiner, T., De Felici, M., Walsh, C.P., 2005. DNA methyltransferase expression in the mouse germ line during periods of de novo methylation. Dev. Dyn. 232, 992–1002.

Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R., Li, E., 1996. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122, 3195–3205.

Li, E., 2002. Chromatin modification and epigenetic reprogramming in mammalian development. Nat. Rev. Genet. 3, 662–673.

Li, E., Bestor, T.H., Jaenisch, R., 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69, 915–926.

Li, J.Y., Lees-Murdock, D.J., Xu, G.L., Walsh, C.P., 2004. Timing of establishment of paternal methylation imprints in the mouse. Genomics 84, 952–960.

Lucifero, D., Mertineit, C., Clarke, H.J., Bestor, T.H., Trasler, J.M., 2002. Methylation dynamics of imprinted genes in mouse germ cells. Genomics 79, 530–538.

Maclean, J.A., Wilkinson, M.F., 2005. Gene regulation in spermatogenesis. Curr. Top. Dev. Biol. 71, 131–197.

Margot, J.B., Ehrenhofer-Murray, A.E., Leonhardt, H., 2003. Interactions within the mammalian DNA methyltransferase family. BMC Mol. Biol. 4, 7.

Mertineit, C., Yoder, J.A., Taketo, T., Laird, D.W., Trasler, J.M., Bestor, T.H., 1998. Sex-specific exons control DNA methyltransferase in mammalian germ cells. Development 125, 889–897.

Mortusewicz, O., Schermelleh, L., Walter, J., Cardoso, M.C., Leonhardt, H., 2005. Recruitment of DNA methyltransferase I to DNA repair sites. Proc. Natl. Acad. Sci. U. S. A. 102, 8905–8909.

Okano, M., Xie, S., Li, E., 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat. Genet. 19, 219–220.

Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247–257.

Reik, W., Walter, J., 2001. Genomic imprinting: parental influence on the genome. Nat. Rev., Genet. 2, 21–32.

Romrell, L.J., Bellve, A.R., Fawcett, D.W., 1976. Separation of mouse spermatogenic cells by sedimentation velocity. A morphological characterization. Dev. Biol. 49, 119–131.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., Hirohashi, S., 2002. Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc. Natl. Acad. Sci. U. S. A. 99, 10060–10065.

Sakai, Y., Suetake, I., Itoh, K., Mizugaki, M., Tajima, S., Yamashina, S., 2001. Expression of DNA methyltransferase (Dnmt1) in testicular germ cells during development of mouse embryo. Cell Struct. Funct. 26, 685–691.

Sakai, Y., Suetake, I., Shinozaki, F., Yamashina, S., Tajima, S., 2004. Co-expression of de novo DNA methyltransferases Dnmt3a2 and Dnmt3L in gonocytes of mouse embryos. Gene Expression Patterns 5, 231–237.

Szabo, P.E., Mann, J.R., 1995. Biallelic expression of imprinted genes in the mouse germ line: implications for erasure, establishment, and mechanisms of genomic imprinting. Genes Dev. 9, 1857–1868.

Szabo, P.E., Hubner, K., Scholer, H., Mann, J.R., 2002. Allele-specific expression of imprinted genes in mouse migratory primordial germ cells. Mech. Dev. 115, 157–160.

Trasler, J.M., Alcivar, A.A., Hake, L.E., Bestor, T., Hecht, N.B., 1992. DNA methyltransferase is developmentally expressed in replicating and non-replicating male germ cells. Nucleic Acids Res. 20, 2541–2545.

Ueda, T., Abe, K., Miura, A., Yuzuriha, M., Zubair, M., Noguchi, M., Niwa, K., Kawase, Y., Kono, T., Matsuda, Y., Fujimoto, H., Shibata, H., Hayashizaki, Y., Sasaki, H., 2000. The paternal methylation imprint of the mouse H19 locus is acquired in the gonocyte stage during foetal testis development. Genes Cells 5, 649–659.

Walsh, C.P., Chaillet, J.R., Bestor, T.H., 1998. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat. Genet. 20, 116–117.

Watanabe, D., Suetake, I., Tada, T., Tajima, S., 2002. Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech. Dev. 118, 187–190.

Watanabe, D., Suetake, I., Tajima, S., Hanaoka, K., 2004. Expression of Dnmt3b in mouse hematopoietic progenitor cells and spermatogonia at specific stages. Gene Expression Patterns 5, 43–49.

Webster, K.E., O'Bryan, M.K., Fletcher, S., Crewther, P.E., Aapola, U., Craig, J., Harrison, D.K., Aung, H., Phutikanit, N., Lyle, R., Meachem, S.J., Antonarakis, S.E., de Kretser, D.M., Hedger, M.P., Peterson, P., Carroll, B.J., Scott, H.S., 2005. Meiotic and epigenetic defects in Dnmt3L-knockout mouse spermatogenesis. Proc. Natl. Acad. Sci. U. S. A. 102, 4068–4073.

Weisenberger, D.J., Velicescu, M., Preciado-Lopez, M.A., Gonzales, F.A., Tsai, Y.C., Liang, G., Jones, P.A., 2002. Identification and characterization of alternatively spliced variants of DNA methyltransferase 3a in mammalian cells. Gene 298, 91–99.

Weisenberger, D.J., Velicescu, M., Cheng, J.C., Gonzales, F.A., Liang, G., Jones, P.A., 2004. Role of the DNA methyltransferase variant DNMT3b3 in DNA methylation. Mol. Cancer Res. 2, 62–72.

Yoder, J.A., Walsh, C.P., Bestor, T.H., 1997. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet. 13, 335–340.